Scientia Horticulturae 173 (2014) 29–36

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Comparative physiological analysis of lotus (*Nelumbo nucifera*) cultivars in response to salt stress and cloning of *NnCIPK* genes

Ruijie Liu[a,b], Haotao Shi[a], Yanping Wang[a], Sha Chen[c], Jiao Deng[a,b], Yanling Liu[a], Shaohua Li[a], Zhulong Chan[a,*]

[a] *Key Laboratory of Plant Germplasm Enhancement and Specialty Agriculture, Wuhan Botanical Garden, Chinese Academy of Sciences, Wuhan 430074, China*
[b] *University of Chinese Academy of Sciences, Beijing 100039, China*
[c] *Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, No. 16, Nanxiaojie, Dongzhimennei, Beijing 100700, China*

ARTICLE INFO

Article history:
Received 6 March 2014
Received in revised form 22 April 2014
Accepted 26 April 2014
Available online 18 May 2014

Keywords:
Antioxidant enzyme
CIPK
Nelumbo nucifera
Reactive oxygen species
Salt stress
Phylogenetic analysis

ABSTRACT

Saline or salt water in the ocean accounts for 96.5% of total fresh water resource in the planet. Salinity is a global social and economic problem which severely inhibited plant growth and development. Utilization of marginal salt affected land and/or water resource becomes increasingly important because of the explosion of world population and climate change. In this study, salt stress resistance of fifteen *N. nucifera* cultivars was firstly evaluated. The results showed that Welcoming Guests was the most resistant cultivar, while Hunan Lotus was the most sensitive one. Resistant cultivar Welcoming Guests accumulated significant higher amount MDA and proline than Hunan Lotus prior to salt stress treatment, indicating Welcoming Guests was pre-conditioned to salt stress. Salt sensitive lotus cultivar exhibited relative lower antioxidant enzyme activities and higher reactive oxygen species accumulation than resistant one after salt treatment. Since calcineurin B-like protein interaction protein kinase (CIPK)/SALT OVERLY SENSITIVE2 gene family played essential roles during plant salt stress response, three *NnCIPK* genes were successfully cloned in this study. Phylogenetic analysis showed that these genes were high homologous to Arabidopsis and grape *CIPK* genes. Expression level analysis indicated that *NnCIPK6* was highly induced by NaCl treatment in resistant cultivar, while expression levels of *NnCIPK14s* showed fluctuation in susceptible cultivar after salt treatment. These results partially characterized mechanisms of lotus salt stress resistance and provided useful information for utilization of lotus cultivars in salt water.

1. Introduction

During the course of their life cycle, plants encounter numerous harsh environmental conditions and have developed various resistance strategies to cope with biotic and abiotic stresses. Salinity is a globally social and economic problem. Approximately 75% of the Earth's surface is surrounded by water and the Earth is therefore referred to as the "blue planet". However, the vast bulk of the water on Earth is regarded as saline or salt water in the ocean which accounts for 96.5% of total water resource in the planet with an average salinity of 35‰ (Shiklomanov, 1993a,b). This salt solution has affected, and continues to affect, the land on which plants are, or might be, grown. The area of salt affected land is already more than 900×10^6 ha, comprising nearly 7% of the world's total land area and one-third of irrigated land, which is sufficient to pose a threat to agriculture (Flowers and Yeo, 1995; Shabala and Cuin, 2008). Salt stress involves a combination of dehydration or osmotic-related stress effects, and damage due to excess sodium ions (Hasegawa et al., 2000). Osmotic stress reduces the ability of plants to take up water and minerals. It not only reduces the growth rate in proportion to the salinity level, but also the tiller numbers in plants (Husain et al., 2003; Munns et al., 2006). Ion toxicity inhibits a variety of processes such as K^+ sorption, vital enzyme reactions, protein synthesis and photosynthesis (Hall and Flowers, 1973; Murguia et al., 1995). Secondary effects include the production of reactive oxygen species (ROS). As important signaling molecules, ROS including H_2O_2 and $O_2^{\bullet -}$ was induced immediately after stress treatment (Shi et al., 2013a,b; Wang et al., 2013a). The over-production of H_2O_2 can lead to oxidative damages by oxidizing proteins, damaging nucleic acids and causing lipid peroxidation.

Abbreviations: CAT, catalase; CIPK, calcineurin B-like (CBL) protein interaction protein kinase; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; $O_2^{\bullet -}$, superoxide radical; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; SOS, salt overly sensitive.

* Corresponding author. Tel.: +86 27 87510823; fax: +86 27 87510251.
E-mail address: zhulongch@wbgcas.cn (Z. Chan).

http://dx.doi.org/10.1016/j.scienta.2014.04.032
0304-4238/

Accordingly, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) were activated to scavenge the over-production of ROS and protect plant cells from ROS caused damage (Apel and Hirt, 2004; Mittler, 2002; Shi et al., 2012).

Plant adaptations to salt stress include avoidance by reduced sodium uptake, sequestration of toxic sodium ions away from the cytoplasm, or production of compatible solutes or osmoprotectants to reduce molecular disruption (Zhu, 2001; Loescher et al., 2011). Natural variations of plants in response to salt stress have been extensively studied in Arabidopsis and other plants (Chan et al., 2013; Cai et al., 2014; Sabra et al., 2012; Wang et al., 2013a). Many genes involved in stress response, carbohydrate biosynthesis, and hormone metabolism showed significant changes between resistant and sensitive varieties (Chan et al., 2013; Wang et al., 2013a), indicating constitutively activated stress responses in salt resistant plant materials. The pioneered work for identification of salt resistance determinants using forward genetics in Arabidopsis has been achieved in the Zhu laboratory (Halfter et al., 2000; Liu and Zhu, 1998; Shi et al., 2000; Zhu, 2000). Genetic and physiological data revealed that SOS3 (SALT OVERLY SENSITIVE3), SOS2, and SOS1 are components of a signal pathway that regulates ion homeostasis and salt resistance. The functions of SOS pathway are Ca^{2+} dependent (Hasegawa et al., 2000; Zhu, 2000). In plants, many Ca^{2+}-sensing protein kinases including CIPK (calcineurin B-like [CBL] protein interaction protein kinase) (Shi et al., 1999; Batistic and Kudla, 2004) have been reported for their involvement in the stress responses. Activated CIPKs can subsequently transduce calcium signals by phosphorylating downstream signaling components (Liu et al., 2000). The first genetically defined CIPK was SOS2 (CIPK24), which interacts with CBL4 (SOS3) under salt-stress conditions, and in turn activates a plasma membrane-localized Na^+/H^+ antiporter (SOS1) and vacuolar H^+-ATPase to promote salt resistance (Liu et al., 2000; Qiu et al., 2002; Batelli et al., 2007). SOS2 is a Serine/Threonine protein kinase with an N-terminal catalytic domain similar to SNF1/AMPK, a C-terminal regulatory domain that is unique to CIPK family kinases, and a FISL (also known as NAF) motif containing 21 amino acids. Functions of both the N-terminal and C-terminal domains have been well characterized as essential domains for salt tolerance, while the FISL motif acts as an autoinhibitory domain to keep the kinase activity inactive by interacting with the kinase domain under normal conditions (Albrecht et al., 2001). Functional analyses of other CIPKs in non-model plant species also exhibited key roles of CIPKs during plant salt stress responses. These genes were induced by salt and overexpression of *CIPK*s enhanced plant salt stress resistance (Tripathi et al., 2009; Wang et al., 2012; Zhang et al., 2013a; Zhou et al., 2014).

Sacred lotus (*Nelumbo nucifera* Gaertn.) is a large aquatic herb with high ecological, ornamental and economic value. Lotus cultivars are classified into three groups: rhizome lotus, seed lotus and ornamental lotus according to their use and morphological features. It is not only used for food and ornamentals, but also used as a source of herbal medicine (Guo, 2009; Han et al., 2007; Borgi et al., 2007). As an economically important aquatic plant, production of lotus has already been affected with increasing concentration of salinity in soil. Commonly cultivated species of lotus are sensitive to salt stress. Therefore, screening of salt resistant species and functional identification of salt-resistance genes from wild type species is expected to improve salt resistance of cultivated species. Cheng et al. (2013) reported a bZIP transcription factor isolated from a salt-resistant *N. nucifera* root using cDNA-AFLP approach. Transgenic tobacco plants exhibited higher resistance to salt treatment. Overexpression of a *N. nucifera* homologue of phytochelatin synthase in Arabidopsis also exhibited increased resistance to cadmium treatment (Liu et al., 2012). These results indicated that isolation of stress responsive genes from *N. nucifera* provided a new approach to improve plant salt stress resistance. Recently, sacred lotus's genome sequence has been released because of interesting traits such as seed longevity and adaptation to the aquatic environment (Ming et al., 2013; Wang et al., 2013b). The availability of genome sequence is critical for functional analysis of gene homologue and identification of novel stress responsive genes in *N. nucifera*.

To date, salt stress resistance of lotus cultivars has not been characterized and the detailed mechanisms remain unknown. In this study, salt resistance of fifteen *N. nucifera* cultivars was evaluated for the first time. Contents of reactive oxygen species (ROS) and activities of several antioxidant enzymes were further compared between salt resistant and salt sensitive cultivars. Because of key roles of SOS2/CIPK gene family during plant salt stress response, full length sequences of three *NnCIPK*s from lotus were successfully cloned and their expression levels were examined in the presence and absence of NaCl. All these data partially depicted the mechanisms of lotus salt response.

2. Materials and methods

2.1. Plant materials and treatments

Totally fifteen varieties of Lotus seeds were obtained from Wuhan Botanical Garden, China, including #1, Red Chrysanthemum; #2, Red Lotus; #3, White Crane; #4, White Pigeon; #5, Hunan Lotus; #6, Hongjian Lotus; #7, Fujian White; #8, Embroidery Jade; #9, Tropical lotus; #10, Little Versicolor; #11, Thousands Red; #12, Welcoming Guests; #13, East-lake Asaka; #14, West-lake Red Lotus; and #15, Chinese Antique. The top part of the blunt end of the seeds was pierced by a pair of pliers before the experiment, which allows water to enter the seeds. Then the seeds were incubated in distilled water at 25 °C under 16 h day/8 h night conditions in the growth chamber for germination until the embryonic axis appeared. Thereafter, three seeds with the same length of embryonic axis were treated with 0 or 200 mM NaCl solution in the plate. Samples collected for the following physiological parameter measurements and RNA isolation were immediately frozen in liquid nitrogen and stored at −80 °C until used. The whole experiment was repeated three times.

2.2. Germination and embryonic axis elongation assays

The length of embryonic axis of lotus was measured and photographed every other day, until the eighth day (0, 2, 4, 6, 8 d). The relative embryonic axis growth was calculated as the following: (embryonic axis length at 8th day after treatment − embryonic axis length before treatment)/(embryonic axis length at 8th day without treatment − embryonic axis length before treatment).

2.3. MDA and proline contents

Malonaldehyde (MDA) was extracted with the trichloroacetic acid (TBA) reaction and was determined at 450, 532 and 600 nm as described by Dhindsa et al. (1981).

The content of proline was determined as described by Bates et al. (1973). Generally, 1 g sample was fully extracted in 10 mL 3% (w/v) sulfosalicylic acid. Then the extractions were added to the mixtures of 2 mL ninhydrin reagent and 2 mL glacial acetic acid and boiled at 100 °C for 30 min. The mixtures were centrifuged at 12,000 × *g* for 5 min after the mixture was cooled to room temperature. The proline levels were determined at 520 nm of absorbance. Simultaneously, purified proline was used as standard.

2.4. In vivo detection of H_2O_2 and O^{2-}

The H_2O_2 content was detected according to method described by Hu et al. (2012). Generally, 1 mL supernatant was mixed with